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(71) Applicant: GIST-BROCADES N.V.
Wateringseweg 1 P.O. Box 1
NL-2600 MA Delft (NL)
Applicant: PLANT GENETIC SYSTEMS, N.V.
Kolonel Bourgstraat 106 Bus 6
B-1040 Brussel (BE)

(72) Inventor: Lambeir, Anne-Marie Virginie Renee
Sparrendreef 30, bus 43
B-3030 Heverlee (BE)
Inventor: Quax, Wilhelmus Johannes
Jan van Galenlaan 8
NL-2253 VB Voorschoten (NL)
Inventor: Lasters, Ignace
Lutgardisstraat 24
B-3081 Perk (BE)
Inventor: Van der Laan, Jan Metske
Korte Straat 13
NL-9718 PE Groningen (NL)

(74) Representative: Huygens, Arthur Victor, Dr. et al
c/o Gist-Brocades N.V. Patent & Trademarks Department Wateringseweg 1 PO Box 1
NL-2600 MA Delft (NL)

(54) Novel glucose isomerases having altered substrate specificity.

(57) A method for selecting amino acid residues is disclosed which upon replacement will give rise to an enzyme with an altered substrate specificity. New mutant glucose isomerases with an altered substrate specificity are provided according to this method. These altered properties are useful in starch degradation and in other sugar conversion reactions.

EP 0 440 273 A2

NOVEL GLUCOSE ISOMERASES HAVING ALTERED SUBSTRATE SPECIFICITY

TECHNICAL FIELD

The present invention relates to the application of protein engineering technology to improve the properties of enzymes. Specifically, the present invention discloses a method for selecting amino acids which upon substitution give rise to an altered substrate specificity. The method is applied to glucose isomerases. In another aspect the invention provides glucose isomerases with an altered substrate specificity. These new glucose isomerases can advantageously be used in industrial processes, for example in the production of high fructose corn syrups (HFCS).

BACKGROUND OF THE INVENTION

Glucose isomerases catalyze the reversible isomerization of glucose to fructose. Fructose is nowadays commonly applied as sugar substitute due to its higher sweetness compared to e.g. sucrose and glucose.

Many microorganisms are known to produce glucose isomerase, see for example the review articles by Wen-Pin Chen in Process Biochemistry, 15 June/July (1980) 30-41 and August/September (1980) 36-41, in which a large number of microorganisms, capable of producing glucose isomerase, are listed.

Several microorganisms can be used for the industrial production of glucose isomerases, among these Streptomyces Ampullariella and Actinoplanes are well known. The Wen-Pin Chen reference describes culture conditions for the microorganisms and recovery and purification methods for the produced glucose isomerases.

Generally the naturally occurring glucose isomerases also show a high affinity for sugars other than glucose. In this respect D-xylose, D-ribose, L-arabinose, D-allose and 6-deoxyglucose were found to be substrates of this enzyme. The K_m values of D-glucose, D-xylose and D-ribose, were shown to vary from microorganism to microorganism and were reported to be in the range, of 0.086-0.920, 0.005-0.093 and 0.35-0.65 M, respectively.

The K_m values for xylose are significantly lower than for glucose, which implies that the correct name for the enzyme is in fact xylose isomerase. Furthermore, the V_{max} of the commonly used glucose isomerases is higher on xylose than on glucose, which also suggest that xylose isomerase is a better name.

Since glucose isomerase is active on different substrates it may be advantageous to alter the substrate specificity depending on the desired reaction product, the specific process in which it is used or the wish to avoid unwanted side-products.

For the application of glucose isomerase in HFCS production a higher V_{max} and a lower K_m on glucose would be useful properties, since the reaction time and the enzyme costs would be reduced.

Another application of glucose isomerase is in the conversion of xylose to ethanol (Jeffries, T.W., Trends Biotechnol. 3 (1985) 208). A higher activity (V_{max}) on xylose and/or a better affinity for xylose would be useful properties for this application.

The digestibility and the taste of feed for monogastric animals can be improved if glucose is converted enzymatically into fructose. In practice the application of glucose isomerase is hampered by the xylose isomerisation activity, which causes an unwanted formation of xylulose in feed. A glucose isomerase with no or reduced specificity (V_{max} or K_m) for xylose would be preferred for application in feed pretreatment.

Clearly, there is a need for altering the substrate specificity of glucose isomerases which would at the same time widen the field of the application of this enzyme.

Recently redesigning of the specific activity of enzymes with the aid of protein engineering techniques has been described. Wells et al. (Proc. Natl. Acad. Sci. USA 84 (1987) 5167) show an example for subtilisin. Bacillus licheniformis and B. amyloliquefaciens serine proteases differ by 31% (86 amino acid residues) in protein sequence and by a factor of 60 in catalytic efficiency on certain substrates. By substituting 3 of the 86 different amino acids from the B. amyloliquefaciens sequence by the corresponding B. licheniformis residues the catalytic activity of the mutant enzyme was improved nearly 60 fold.

In another paper it is described how a lactate dehydrogenase was changed into a malate dehydrogenase by mutating glutamine 102 into arginine 102 (Wilks et al., Science 242 (1988) 1541).

In both cases referred to above, serine protease and lactate dehydrogenase, the modification proposal was based on the comparison of the molecule to be modified and naturally occurring enzymes, which already showed the desired substrate specificity. In the same way the specificity of cytochrome $p450_{15\alpha}$ was changed into the specificity of cytochrome $p450_{coh}$ by replacing Leu209 with Phe209 (Lindberg and Negishi, Nature 339 (1989) 632).

For glucose isomerase no naturally occurring enzyme is known, which shows a better specificity for glucose

than for xylose. The above-mentioned method, based on the comparison of active sites of homologous enzymes having a different substrate specificity, can therefore not be applied to glucose isomerase.

WO 89/01520 (Cetus) lists a number of muteins of the xylose isomerase which may be obtained from Streptomyces rubiginosus and that may have an increased stability. The selection of possible sites that may be mutated is based on criteria differing from the ones used in the present invention. More than 300 mutants are listed but no data are presented concerning the characteristics and the alterations therein of the mutant enzyme molecules.

SUMMARY OF THE INVENTION

The present invention discloses a method for selecting amino acids which upon replacement lead to an altered substrate specificity of a given enzyme. This method, which is generally applicable, is used to alter the substrate specificity of glucose isomerase.

Thus, the invention also provides glucose isomerases with altered substrate specificities. This altered substrate specificity is expressed in terms of an altered substrate binding capacity and/or an altered catalytic activity. Specifically, mutant glucose isomerases are provided with both an absolute and a relative change in substrate binding capacity and catalytic activity on glucose and xylose as substrates.

Furthermore, mutant glucose isomerases are provided which show that parameters, such as, increased stability (expressed in terms of decay constant) and altered substrate specificity can be combined into a single molecule by adding the respective mutations that have these effects.

The mutant glucose isomerases are obtained by the expression of a gene encoding said glucose isomerase enzyme having an amino acid sequence which differs at least in one amino acid from the wildtype glucose isomerase enzyme.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows a schematic representation of the active site of glucose isomerase from Actinoplanes missouriensis, derived from the three dimensional structure of the glucose isomerase - xylitol complex. The inhibitor is shown in full detail in the centre of the figure. For the amino acid residues only those atoms are drawn which are involved in hydrogen bonding. Residue names are in boxes drawn with solid lines, solvent molecules in boxes drawn with dashed lines. Metal binding sites are represented by ovals numbered 395 and 580. Dashed lines indicate electrostatic interactions : the thin dotted lines represent hydrogen bonds, the fat dashed lines the proposed ligation of the metals.
Strictly conserved residues are marked by an asterix. For non-conserved residues the substitutions found in nature are indicated.

Figure 2 shows the alignment of the amino acid sequences of glucose isomerases obtained from different microorganisms. The complete sequence of Actinoplanes missouriensis glucose isomerase is given. The amino acid sequence of Ampullariella glucose isomerase differs from that of the published sequences (Saari, J. Bacteriol., 169, (1987) 612) by one residue : Proline 177 in the published sequence was found to be Arginine.

The Streptomyces thermovulgaris sequence has only been established upto amino acid 346. Undetermined residues are left blank. A dot indicates the absence of an amino acid residue at this position as compared to any of the other sequences. The different species are indicated by the following symbols :

Ami. : Actinoplanes missouriensis DSM 4643
Amp. : Ampullariella species ATCC 31351
Svi. : Streptomyces violaceoruber LMG 7183
Smu. : Streptomyces murinus
Sth. : Streptomyces thermovulgaris DSM 40444
Art. : Arthrobacter species
Bsu. : Bacillus subtilis
Eco. : Escherichia coli
Lxy. : Lactobacillus xylosus

The secondary structure assignment was made in the structure of Actinoplanes missouriensis. Helices in the barrel are enclosed by solid lines. Shaded boxes indicate β-strands.

Figure 3 shows a comparison of the H290N mutant (bold) with the wildtype-sorbitol-Mg structure.

DETAILED DESCRIPTION OF THE INVENTION

The present invention discloses a method for selecting amino acids, in an enzyme, that upon substitution

give rise to an altered substrate specificity. To obtain such a mutant enzyme the DNA seqence encoding this enzyme is altered in such a way that the encoded enzyme has one or more selected amino acid replacements. The altered DNA is expressed in a desired host/vector system.

To select appropriate (point) mutations a rational approach is taken, relying on the well coordinated application of protein crystallography, molecular modelling and computational methods, enzymology and kinetics, molecular biology and protein chemistry techniques. The strategies for the identification of target amino acid sites are innovative in a sense that it is recognized that point mutations rarely cause local perturbations, but affect several different properties of the protein structure at once, causing a change in different properties as well. Therefore, although the described strategies make use of well established structure-function relationships, they also provide a rational way to avoid or correct unwanted alterations of kinetic and structural properties.

Alteration of substrate specificity by replacement of appropriate amino acids

To modulate the substrate binding to the active site of an enzyme, the following general rules can be applied in sequential order, provided that data are available concerning the Three-Dimensional (3D) structure for the enzyme-substrate or enzyme-substrate-analogue/inhibitor complexes :

a) select all residues and crystallographically assigned water molecules which have at least one atom within a sphere of 4Å surrounding the atoms of the substrate or of a substrate analogue/inhibitor bond in the active site ;

b) select all the residues which are in Van der Waals contact with the residues and water molecules obtained by application of criterion a) ;

c) discard from the recorded list of residues and water molecules those that are implied in catalysis, cofactor binding (such as metal ions and nucleotides) and essential intersubunit interactions (in the case of oligomeric enzymes) ;

d) discard those residues and water molecules that interfere with the structural role of the above-selected residues. Model building and analysis of the conserved nature of the target residues can be used to identify an essential structural role ;

e) to modulate the substrate affinity, one or more of the above-selected residues can be substituted by alteration of the genetic code to change one or more of the following interactions and properties :

- e1) steric hindrance through altering the residue size ;
- e2) hydrophobicity/polarity of the substrate surroundings ;
- e3) solvation of the substrate surroundings either by providing side chains that solvate via hydrogen bonding to groups within the substrate or through substitutions that alter the water distribution within the surroundings of the substrate ;
- e4) flexibility of individual residues, segments or the overall protein structure by substitutions that disrupt hydrogen bonding networks, decrease the local packing density in the surroundings of the substrate or introduce cavities.

The above set of rules is a general one which can in principle be applied to all enzymes provided that enough structural data are available. In order to demonstrate the feasibility of this set of rules a specific example will be discussed below.

Application of the general rules to modulate the substrate specificity of Glucose Isomerase

Although the selection of residues by applying criteria a) through e) as given above will be demonstrated here using the specific example of Actinoplanes missouriensis glucose isomerase, it is clear that due to extensive homology similar substitution sites can be selected in glucose isomerases obtained from other species. The mentioned sequence homology is demonstrated in Figure 2 which gives an alignment of glucose isomerases obtained from ; Actinoplanes missourienses, Ampullariella species, Streptomyces violaceoruber, Streptomyces murinus, Streptomyces thermovulgaris, Arthrobacter species, Bacillus subtilis, Escherichia coli and Lactobacillus xylosus. The approach described above would also give rise, after amino acid replacement at corresponding positions in the glucose isomerases from the other species, to an altered substrate specificity of other glucose isomerases.

In general, it can be assumed that where the overall homology is greater than 65%, preferably greater than 74% (minimal homology between Actinoplanes missouriensis and Streptomyces glucose isomerase, according to Amore and Hollenberg, Nucl. Acids Res. 17, 7515 (1989)), and more preferably greater than 85% and where the 3D structure is similar, amino acid replacements will lead to similar changes in substrate specificity. With similar changes in substrate specificity we mean the direction in which the kinetic parameters change and not the magnitude. Specifically one expects the glucose isomerases from species belonging to the order of the

Actinomycetales to have such a high degree of similarity that the alteration of substrate specificities due to amino acid replacements at the selected sites are similar. Actinoplanes missouriensis is the preferred source of glucose isomerase to mutate.

Changes in substrate specificity according to the present invention include all combinations of increase and decrease of V_{max} and K_m for both glucose and xylose. A person skilled in the art will understand that this encompasses the changes in other kinetic parameters. Furthermore, the specificities for other substrates will inherently be changed also. The proposed rules for changing the substrate specificity are not restricted to the mentioned substrates, they can be applied to other substrates. Among these are, D-ribose, L-arabinose, D-allose and 6-deoxyglucose.

Thus apart from providing a general method for altering the substrate specificity the present invention applies this method to glucose isomerase.

The selected amino acid replacements can be engineered in the DNA encoding the glucose isomerase by methods well known to a person skilled in the art (e.g. site-directed mutagenesis). The DNA encoding the glucose isomerase or its mutants may be cloned on an expression vector and this construct may be transformed to a host wherein the gene is expressed, the mRNA translated and preferably the mature protein, or a precursor, secreted from the cell. Subsequently the protein can be purified. Standard procedures can be found in Maniatis et al. (Cold Spring Harbor, 1st and 2nd edition, 1982 and 1989 respectively).

The application of these methods gives rise to mutant glucose isomerase enzymes, obtained by the expression of a gene encoding said glucose isomerase enzyme having an amino acid sequence which differs at least in one amino acid from the wildtype glucose isomerase enzyme, which mutant glucose isomerase enzyme is characterized in that it exhibits an altered substrate specificity. Instead of single mutants also double mutants may be obtained. Some of these double mutants, aimed at combining the desired properties, show that the properties are at least partially cumulative.

Examples are provided, in the present application, of mutants with both an increased specificity for glucose and an increased stability.

The substrate specificity of the new enzymes can be tested on the substrate that is necessary for the desired application. Here special attention is paid to the kinetic parameters concerning glucose and xylose as a substrate and also to the relative changes in these parameters.

Figure 1 shows a schematic map of residues around the active site of A. missouriensis. In the following methods are described to design mutants which have a higher relative specificity for glucose compared to the wildtype enzyme.

Applying the rules given above to glucose isomerase from Actinoplanes missouriensis and using the coordinates of the sorbitol-Gl-Co^{2+} structure we obtain the following list of residues :

- Residues and waters selected by criterion (a) : 16Trp, 54His, 88Met, 90Thr, 94Phe, 135Val, 137Trp, 181Glu, 183Lys, 217Glu, 220His, 245Asp, 255Asp, 292Asp, 294Lys, 26Phe and 4 water molecules that are arbitrarily denoted as 491, 492, 690 and 887.
- Additional residues were selected by criterion (b). In this case all residues within a sphere of 2 Å around the above mentioned residues (criterion (a)) were taken.

Giving the following amino acids :

15Leu, 17Thr, 20Trp, 25Ala, 27Gly, 52Thr, 53Phe, 55Asp, 57Asp, 87Pro, 89Val, 91Thr, 92Asn, 93Leu, 95Thr, 133Thr, 134Leu, 136Leu, 138Gly, 140Arg, 179Ala, 180Ile, 182Pro, 184Pro, 186Glu, 215Asn, 216Pro, 218Thr, 219Gly, 221Glu, 243His, 244Ile, 246Leu, 254Phe, 256Gln, 257Asp, 290His, 291Phe, 293Tyr and 295Pro.

- The catalytic residues : 54His, 183Lys, 220His ; the cation ligands : 181Glu, 217Glu, 221Glu, 245Asp, 255Asp, 292Asp, water 690 and the interface residue : 26Phe ; are discarded by applying criterion (c).
- Residues 16Trp, 94Phe and 137Trp form a conserved hydrophobic cluster determining the overall shape of the substrate binding pocket and they are therefore discarded by applying criterion (d). Furthermore residue 184Pro is discarded by criterion (d).

The preferred residues to be substituted resulting after application of the above mentioned criteria are shown in Table 1.

Table 1

15Leu	17Thr	20Trp	25Ala	27Gly
52Thr	53Phe	55Asp	57Asp	87Pro
88Met	89Val	90Thr	91Thr	92Asn
93Leu	95Thr	133Thr	134Leu	135Val
136Leu	138Gly	140Arg	179Ala	180Ile
182Pro	186Glu	215Asn	216Pro	218Thr
219Gly	243His	244Ile	246Leu	254Phe
256Gln	257Asp	290His	291Phe	293Tyr
294Lys	295Pro			

Single substitutions at these sites and/or combinations of substitutions at the different sites may lead to an improved activity either through relief of steric hindrance or by the modulation of polarity of the sugar environment. Different properties such as modulating the affinity and/or catalytic efficiency for various substrates may be altered in this way.

In the specific case of increasing the specificity for glucose relative to xylose it is important to note that the substrate specificity for wildtype glucose isomerase decreases going from xylose to 6-deoxy-glucose to glucose. The inhibition constant for sorbitol is larger than for xylitol. This observation suggests that steric hindrance upon binding of the larger substrate or inhibitor contributes to the lower specificity. In the crystal structure of the enzyme-sorbitol-cobalt complex from A. missouriensis the following residues are found within 4Å of the hydroxyl group attached to the C6 of the sorbitol : 16Trp, 88Met, 135Val, 90Thr and a water molecule denoted 491. The hydrophobic side chains 16Trp, 88Met, 135Val are unable to solvate the hydroxyl group. The hydroxyl group of the 90Thr side chain is rotated away, exposing its methyl group to the inhibitor. The water molecule (491) bridges O6 and O4 of the sorbitol. In the structure of the enzyme-cobalt complex determined in the absence of substrate or inhibitor, the active site contains a number of water molecules bound at positions roughly corresponding to the positions of the O1, O2, O3, O4 and O5 of the inhibitor in the enzyme-sorbitol-cobalt complex. However, the environment corresponding to the O6 position is not able to accommodate a water molecule. This provides additional evidence that the hydroxyl group on C6 is not properly solvated.

The substitutions which have been made in the collection of target residues (by applying criteria a - e) were aimed to increase the polarity of the environment of the substrate hydroxyl groups, particularly of the 06, and to increase the flexibility of the active site to accommodate the larger substrate.

Amongst others the following single and combined point mutations have been made, leading to the intended changes in glucose specificity.

25Ala to Lys : introduction of a positively charged residue at a distance of 6 to 8 Å of the O1, O2 and O3 of the substrate. Disruption of the water structure in the interface. Displacement of 26Phe, which shapes the hydrophobic pocket accommodating the C1 aliphatic hydrogens of the substrate.

243His tot Asn : increased flexibility due to changes in the protein-water hydrogen bonding network in the interior of the barrel.

290His to Asn : increased flexibility due to changes in the protein-water hydrogen bonding network in the interior of the barrel, displacement of water.

290His to Asn combined with 253Lys to Arg : the 253Lys to Arg mutation is introduced to increase stability or prevent glycation (EP-A-0351029) which leads to irreversible inactivation.

88Met to Ser : increasing the polarity of the 06 binding pocket, increasing flexibility by locally altering the packing of the protein.

88Met to Ser combined with 243His to Asn : increasing the polarity of the 06 binding pocket, increasing flexibility by locally altering the packing of the protein.

88Met to Ser combined with 290His to Asn : increasing flexibility due to changes in the protein-water hydrogen bonding network in the interior of the barrel, displacement of water, increasing the polarity of the 06 binding pocket.

90Thr to Ser : altered flexibility of the side chain, increasing flexibility by locally altering the packing of the protein, altering the environment of a substrate bound water molecule (492).

90Thr to Ser combined with 135Val to Gln : altered flexibility of the side chain at position 90, increasing flexibility by locally altering the packing of the protein, altering the environment of a substrate bound water molecule (492), replacement of hydrophobic environment by a polar residue, possibility of a direct hydrogen bond between the glutamine and the C6 hydroxyl group.

90Thr to Ser combined with 135Val to Gln and 215Asn to Ser: altered flexibility, replacement of

hydrophobic environment by polar residues, possibility to remove a water molecule, possibility of a direct hydrogen bond between the 90Ser and the C6 hydroxyl group.

135Val to Thr : replacement of hydrophobic environment by a polar residue.

135Val to Gln : replacement of hydrophobic environment by a polar residue, possibility of a direct hydrogen bond between the glutamine and the C6 hydroxyl group.

These mutation proposals are meant to exemplify a rational which can be applied to create novel glucose isomerases with desired specificities. However, it will be clear to those skilled in the art that desired mutants can be obtained by changing target residues into amino acids differing from the mentioned ones in a similar manner.

Replacing, removing, shifting bound water molecules in the active site

From comparison of the sorbitol structure with the xylitol structure it appears that particular water molecules may interfere with proper glucose binding. Replacement of this water by an appropriate amino acid side chain, or moving the position of the water by introducing a shorter side chain at the appropriate position may cause an increase in affinity.

Glucose isomerase mutant H290N, which has an enhanced specificity for glucose (K_m=250 mM, V_{max}=41 µmoles/min/mg) can serve as an example. The removing or shifting of bound water molecules in the active site of mutant H290N is correlated with the observed increase in activity for glucose. Similar effects may be obtained by shifting other bound water molecules. Water molecules which are in a sphere of 4 Å around atoms O3, O4, O5 or O6 of the substrate are candidates to be removed or shifted by mutating neighbouring amino acids.

In the following examples recombinant DNA techniques are applied to introduce point mutations in the gene cloned from Actinoplanes missouriensis. The protein is overexpressed in E. coli, purified and characterized in vitro and in application conditions as described elsewhere (Van Tilburg, 1983, Thesis : "Engineering aspects of biocatalysts in industrial starch conversion", Delftse Universitaire Pers).

EXPERIMENTAL

Cloning and expression of the D-glucose isomerase gene

D-glucose isomerase (GI) is synonymously used for D-xylose isomerase (D-xylose) ketol-isomerase (EC 5.3.1.5), an enzyme that converts D-xylose into D-xylulose. The D-glucose isomerase from Actinoplanes missouriensis produced by engineered E. coli strains is designated as EcoAmi (DSM) GI. To distinguish the Actinoplanes missouriensis gene coding for GI from the analogous E. coli xylA gene, the former will be designated as GI.

Methods for manipulation of DNA molecules are described in Maniatis et al. (1982, Cold Sprong Harbor Laboratory) and Ausubel et al. (1987, Current Protocols in Molecular Biology, John Wiley & Sons Inc. New York). Cloning and DNA sequence of the glucose isomerase gene from Actinoplanes missouriensis DSM 43046 is described elsewhere (EP-A-0351029). The derived amino acid sequence of GI is numbered and compared with other glucose isomerases in Figure 2. In the following, the numbering of amino acids refers to Figure 2.

Wildtype and mutant GI enzymes were produced in E. coli strain K514 as described in EP-A-0351029. EP-A-0351029 specifically describes most of the techniques applied in this application and it is therefore incorporated herein by reference.

Assay of the enzymatic activity of the expression product

The enzymatic activity of glucose isomerase was assayed as described below (1 unit of enzymatic activity produces 1.0 micromole of product -D-xylulose or D-fructose- per minute ; therefore, specific activity -spa- is expressed as units per mg of GI enzymes).

GI activity was assayed directly by measuring the increase in absorbance at 278 nm of xylulose produced at 35°C by isomerisation of xylose by glucose isomerases. This assay was performed in 50 mM triethanolamine buffer, pH 7.5, containing 10mM $MgSO_4$, in the presence of 0.1 M xylose. The final concentration of glucose isomerase in the assay was ± 0.01 mg/ml, this concentration was precisely determined, prior to dilution in the enzymatic assay mixture, by absorption spectroscopy using an extinction coefficient of 1.08 at 278 nm for a solution of enzyme of 1.0 mg/ml.

In the D-Sorbitol Dehydrogenase Coupled Assay, enzymatic determination of D-xylulose was performed at 35°C as previously described (Kersters-Hilderson et al., Enzyme Microb. Technol. 9 (1987) 145) in 50 mM triethanolamine, pH 7.5, 10mM $MgSO_4$, and 0.1 M xylose, in the presence of ± 2 × 10^{-8} M D-sorbitol dehyd-

rogenase (L-iditol : NAD oxido-reductase, EC 1.1.14), and 0.15 mM NADH. The final concentration of glucose isomerase in this assay was ± 2.5 × 10^{-3} mg/ml, this concentration was precisely determined as described above.

With glucose as a substrate GI activity can be assayed by the measurement of D-fructose produced during the isomerization reaction using the cysteine-carbazole method (CCM) which is based on the reaction of ketosugars with carbazole in acids to yield a purple product (Dische and Borenfreund, J: Biol. Chem. 192 (1951) 583). Alternatively, the D-fructose produced during the isomerization reaction can be determined enzymatically using sorbitol dehydrogenase and NADH.

As a measure of specificity the quotient V_{max}/K_m is sometimes used (Wells et al., ibid). For mutants the V_{max}/K_m values for xylose and for glucose were routinely calculated. Measurements for xylose parameters are carried out at 35°C, whereas glucose parameters are determined at 60°C. These temperatures are chosen for practical reasons. To find out whether conclusions about relative specificity are generally applicable independent of the measurement temperature, some measurements on xylose were performed at 60°C for both wildtype and mutant enzymes. It was found that conclusions that could be drawn concerning steady-state kinetic parameters at 60°C were similar to those at 35°C.

EXAMPLES

Example 1 : Mutants with improved catalytic properties

The 3-dimensional structure of Actinoplanes missouriensis glucose isomerase was studied to select those residues which upon change might yield an improved substrate binding, catalytic activity or substrate specificity. Residues directly or indirectly (via another residue or water molecule) within 4 Å from O1-O6 of the substrate were selected and changed by site-directed mutagenesis with the aid of the pMa, pMc vector system (Stanssens et al., Nucl. Acids Res. 17 (1989) 4441)

In Table 2 below the enzymatic parameters of several of these selected mutants are shown :

Table 2

Mutant	V_{max} xy (μmol/min/mg)	V_{max} gl (μmol/min/mg)	V_{max} gl/xy	K_m xy (mM)	K_m gl (mM)	K_m xy/gl x10^{-2}	V_{max}/K_m xy	V_{max}/Km gl
Wild Type	24.20	34.80	1.43	4.80	290	1.7	5.04	0.12
A25K	7.66	22.61	2.95	2.73	207	1.3	2.81	0.11
M88S	19.37	29.44	1.51	7.60	263	2.9	2.55	0.11
M88SH243N	13.90	24.98	1.79	11.92	387	3.1	1.17	0.06
M88SH290N	16.07	49.77	3.09	14.82	406	3.6	1.08	0.12
T90S	37.07	32.77	0.88	16.76	180	9	2.21	0.18
T90SV135Q	0.54	19.30	36	182	2822	6.4	0.00	0.01
V135Q	0.67	5.57	8.3	56.00	1120	5	0.01	0.00
V135T	10.90	33.11	3.04	19.95	678	3	0.55	0.05
E186D	8.51	37.90	4.45	7.00	736	0.9	1.22	0.05
E186Q	0.77	2.06	2.68	1.80	57	3	0.43	0.04
H243N	18.90	22.00	1.16	5.80	180	3.2	3.26	0.12
K253RH290N	26.70	49.13	1.84	12.80	331	3.9	2.09	0.15
L258K	24.23	44.17	1.82	4.44	327	1.4	5.46	0.14
H290N	24.00	41.80	1.74	9.70	250	4	2.47	0.17
K294Q	5.70	9.70	1.70	32.00	309	10	0.18	0.03
K294R	13.80	27.52	4.82	4.50	308	1.5	3.07	0.09
T90SV135Q, N215S	0.93	39.6	42.60	360	1000	36	0.0025	0.04

Conditions for determining enzymatic parameters were as follows:
V_{max}, xylose : 35°C, 10mM $Mg^{2}+$, pH 7.5;
V_{max}, glucose: 60°C, 10mM $Mg^{2}+$, pH 7.5;
K_m, xylose : 35°C, 10mM $Mg^{2}+$, pH 7.5;
K_m, glucose : 60°C, 10mM $Mg^{2}+$, pH 7.5.

Activities were measured on purified enzyme. The coupled sorbitol dehydrogenase assay was used for xylose, the cysteine-carbazole or discontinuous sorbitol dehydrogenase method was used for glucose.

Example 2 : Glucose isomerases with improved affinity for glucose

Table 3 summarizes measured K_m values from various mutants compared to wildtype enzyme. It can be seen that all of these mutants have a lower K_m for glucose. This means that the binding of the substrate glucose is enhanced. Moreover, for some of the mutants the K_m for xylose has not improved but became worse, as exemplified in Table 2. Thus, mutants M88S, T90S, T90SV135Q, V135Q, H290N, K294Q, H243N have acquired a better $K_m$xylose/$K_m$glucose ratio as compared to the wild-type enzyme.

Table 3

MUTANT	K(gl)	K(gl)/WT
E186Q	57.000	0.197
H243N	180.000	0.621
T90S	180.000	0.621
A25K	207.000	0.714
H290N	250.000	0.862
M88S	203.000	0.907
Wildtype	290.000	1.000

The Km for glucose is expressed in mM.

Example 3 : Glucose isomerases with enhanced catalytic activity on glucose

Glucose isomerase mutants with enhanced catalytic activity on glucose are E186D, L258K, H290N and combined mutations with H290N. L258K was not selected using the criteria described in the general methods as the leucine at position 258 is about 10Å away from the substrate or inhibitor.

In Table 4 the V_{max} relative to wildtype V_{max} is shown. An increase from 8,9% to 43% is shown. This will give rise to a faster isomerisation of the preferred substrate glucose.

Table 4

MUTANT	V(gl)	V(gl)/WT
M88SH290N	49.770	1.430
K253RH290N	49.130	1.412
L258K	44.170	1.269
H290N	41.800	1.201
T90SV135QN215S	39.600	1.138
E186D	37.900	1.089
Wildtype	34.800	1.000

The V_{max} for glucose is expressed in micromoles/min/mg.

Mutant H290N shows a V_{max} for glucose of 41.80 which is significantly better than the wildtype enzyme. Moreover, the V_{max}xylose has not improved. The ratio V_{max}glucose/V_{max}xylose of H290N has therefore been

improved, rendering this mutant more into a "true" glucose isomerase.

Example 4 : Structure of H290N glucose isomerase

In the crystal structure of the mutant H290N, it is observed that the amide group of the Asn side chain is superimposable on the imidazole group of w.t. molecule (Fig.3). As a consequence the amide at 290Asn maintains the hydrogen bond to 245Asp. In addition, the Oδ1 atom of 290Asn can hydrogen bond to the hydroxyl of 12Ser.

The hydroxyl group of 52Thr, hydrogen bonded to ne2 of 290His in the w.t. structures, can no longer hydrogen bond to 290Asn. In the mutant the hydroxyl group of 52Thr is rotated (X^1 torsion) in such a way that it hydrogen bonds to a water molecule (wildtype $X^1 = 71°$, mutant $X^1 = -172.5°$). This water molecule present in the mutant structure is positioned as the 88Met sδ atom (distance = 0.45Å) in the wildtype. The introduction of a water molecule at this position, which forms a hydrogen bond to 52Thr, forces a reorientation of the side chain of 88Met in the mutant structure as compared to wildtype. In addition the 52Thr side chain hydrogen bonds in the mutant to the main chain hydrogen of 53Phe (d = 2.23Å).

The reorientation of the 88Met side chain in H290N necessitates the movement of 243His. In addition the water molecule (490), bridging 12Ser to 243His in the wildtype structure, disappears because of steric hindrance with the methyl group of 52Thr and the new orientation of 88Met. 243His adopts another X^1 angle (in w.t. $X^1 = -169°$ and in mutant $X^1 = -77°$) abolishing the hydrogen bond with 215Asn. The space left by the imidazole of 243His is filed with a water molecule (615) which hydrogen bonds to the amide of 215Asn (not shown). In addition, this water molecule hydrogen bonds to a novel water molecule (872) which hydrogen bonds to main chain hydrogen atom of 244Ile.

Additional evidence for the change in flexibility of the side chains in the C6 hydroxyl environment is given by the temperature factors for the residues 52Thr, 88Met and 243His, which is twice as high compared to the wildtype structure.

As a result of the complex rearrangements caused by the H290N mutation, three water molecules are solvating the C6 hydroxyl group of the sorbitol.

This exemplifies that replacing, moving or shifting bond water molecules in the active site can result in desired changes in enzyme activity.

Example 5 : Glucose isomerase with an improved substrate binding

In Table 5 several mutants with improved xylose binding are shown. A25K and E186D have a descreased K_m for both xylose and glucose. E186Q has also a decreased K_m for fructose. Enzymes with high substrate affinity are preferred under conditions of low substrate concentrations.

Table 5

MUTANT	K(xy)	K(gl)	K(xy)/Kgl)	K(xy)/WT	K(gl)/WT
E186Q	1.800	57.000	0.032	0.375	0.197
A25K	2.730	207.000	0.013	0.569	0.714
L258K	4.440	327.000	0.014	0.925	1.128
K294R	4.500	308.000	0.015	0.938	1.062
Wild-type	4.800	290.000	0.017	1.000	1.000

K_m values are expressed in mM.
Note that only E186Q and A25K have an improved affinity for both substrates tested.

Example 6 : Mutant with an improved specificity for glucose

The relative specificity is defined as the V_{max}/K_m ratio for xylose divided by the V_{max}/K_m ratio for glucose. Therefore, if the number is smaller than for the wild-type the relative specificity for glucose is increased.

In Table 6 mutant glucose isomerases with an increased relative specificity for glucose are shown. Mutants V135Q, T90SV135Q and T90SV135QN215S have a largely reduced activity on xylose. Even though in absolute terms the activity with glucose is reduced, the ratio of V_{max}/K_m of glucose over xylose has been improved con-

siderably. Therefore these mutants have changed their specificity to become a real glucose isomerase virtually without xylose isomerase activity.

The combination of mutations T90S, V135Q and N215S into a triple mutant shows that these mutations are additive with respect to the kinetic parameters (Table 6).

Table 6

MUTANT	V/K(xy)	V/K(gl)	V/K(xy)/(gl)	r.spec./WT
T90SV135Q, N215S	0.003	0.040	0.065	0.002
N215S	0.058	0.018	3.193	0.076
T90SV135Q	0.003	0.007	0.434	0.010
V135Q	0.012	0.005	2.402	0.057
K294Q	0.178	0.031	5.674	0.135
M88SH290N	1.084	0.123	8.846	0.211
V135T	0.547	0.049	11.209	0.267
E186Q	0.428	0.036	11.837	0.282
T90S	2.212	0.182	12.138	0.289
K253RH290N	2.086	0.146	14.053	0.334
H290N	2.474	0.167	14.798	0.352
M88SH243N	1.166	0.065	18.066	0.430
M88S	2.549	0.112	22.768	0.542
E186D	1.216	0.051	23.609	0.562
A25K	2.806	0.109	25.688	0.611
H243N	3.259	0.122	26.661	0.635
K294R	3.067	0.089	34.322	0.817
L258K	5.457	0.135	40.401	0.962
Wildtype	5.042	0.120	42.014	1.000

Example 7 : Structural changes occurring in the M88SH243N mutant

Substrate specificity can also be changed by amino acid replacements resulting in amino acids that show a greater side-chain flexibility.

In the crystal structure of the mutant M88SH243N it is observed that the 243Asn and 52Thr adopt multiple conformations, reflecting the increase of flexibility in the side chain environment of the C6 hydroxyl environment of the substrate.

It is also observed that the Cα positions of the adjacent beta barrel strands containing residues 88Ser, 52Thr, 135Val and 177Arg are displaced by 0.3 to 0.5 Å, making the interior of the barrel slightly larger.

The space created by the 88Met to 88Ser mutation is filled with an additional water molecule. The observed alternate side chain locations and the slight movement of the barrel allow the introduction of another water molecule and the movement of a water molecule in the C6 hyrdoxyl direction of the substrate.

Example 8 : Mutants with improved properties in the presence of Mn^{2+}

In addition to Mg^{2+}, Mn^{2+} can be used as a bivalent cation during isomerisation. Although Mn^{2+} is not commonly used in commercial isomerisation processes, its use can be envisaged for applications in which metal ions can be removed from the product or in which metal ions are not relevant for the quality of the product.

Mutant E186Q shows an improved catalytic activity towards glucose in the presence of Mn^{2+}, see Table 7 :

Table 7

E186Q

	V_{max} (μmoles/min/mg)	Km(mM)	Conditions
Xylose	5.4	3.15	35°C, 1mM Mn, pH 7.5
Xylose	14.4	7.83	35°C, 2mM Mn, pH 6.4
Glucose	22.6	387	60°C, 1mM Mn, pH 7.5
Mn	5.63	0.01	35°C,100mM xylose,pH 7.5
Mn	14.5	< 0.03	35°C,200mM xylose,pH 6.4

Wildtype

	V_{max} (μmoles/min/mg)	Km(mM)	Conditions
Xylose	8.6	13.2	35°C, 1mM Mn, pH 7.5
Glucose	6.5	1537	60°C, 1mM Mn, Ph 7.5
Mn	10.2	0.0048	35°C,100mM xylose,pH7.5

The methods used were as in Example 1. The improvement in V_{max}glucose is 3- to 4-fold as compared to the wild-type enzyme. The improvement in $K_m$glucose is 4-fold. Since 186E is in the vicinity of the UP metal position (see Figure 1), it can be envisaged that 186Q is in better concert with the larger Mn^{2+} radius than with the Mg^{2+} radius.

Example 9 : Application testing of the mutant K253RH290N

Mutant H290N shows an increased activity on glucose as can be seen in Table 4. Table 6 shows that the specificity for glucose is also increased in this mutant.
This mutant was immobilized as described in EP-A-351029 (Example 7 of that application). Application testing of the wildtype and this mutant glucose isomerase was performed as described in the same application (Example 8). The stability is indicated by the first order decay constant (K_d, the lower the decay constant the more stable the enzyme). Table 8 gives the K_d values for the wildtype and mutant glucose isomerases.

Table 8

Decay constants for wildtype and mutant glucose isomerase, immobilized on Lewatit

	K_d (x 10^6 sec^{-1})
Wildtype	2.5
H290N	3.1
K253R	0.7
H290NK253R	1.6

As can be seen in Table 8, H290N is destabilized as compared with the wildtype glucose isomerase. K253R was found to stabilize the wildtype glucose isomerase by a factor larger than three. Combination of H290N with the stability mutation K253R shows that these characteristics are additive. Furthermore, it can be seen in Table 4 that the activity of K253RH290N on glucose is not negatively influenced by the stability mutation, on the contrary the double mutant shows an even higher activity on this substrate than mutant H290N. As far as the specificity is concerned, in Table 6 it can be seen that the K253R mutation does not substantially influence the specificity of the H290N mutant.

Thus it can be concluded that activity mutants can be stabilized by introducing mutations that have been shown to stabilize the wildtype enzyme.

It is to be understood that the above mentioned examples are meant to demonstrate the concept of the invention and that they are not meant to limit the scope. In view of this it is clear that combinations of the above mentioned mutations combined with other mutations leading to altered characteristics e.g. thermostability, shifted pH optimum or metal binding are within the scope of the subject invention.

Claims

1. A mutant glucose isomerase enzyme, obtained by expression of a gene encoding said glucose isomerase enzyme having an amino acid sequence which differs at least in one amino acid from the wildtype glucose isomerase enzyme, which mutant glucose isomerase enzyme is characterized in that it exhibits an altered substrate specificity.

2. A mutant glucose isomerase enzyme according to Claim 1, characterized in that it has a V_{max} for glucose, at temperatures between 20 °C and 85 °C, higher than that of the naturally occurring glucose isomerase.

3. A mutant glucose isomerase enzyme according to Claim 1, characterized in that it distinguishes over a naturally occurring glucose isomerase having the same essential biological activity in that it has a K_m for glucose, at temperatures between 20 °C and 85 °C, lower than that of naturally occurring glucose isomerase.

4. A mutant glucose isomerase enzyme according to Claim 1, characterized in that it distinguishes over a naturally occurring glucose isomerase having the same essential biological activity in that it has a V_{max} for xylose, at temperatures between 20 °C and 85 °C, higher than that of the naturally occurring glucose isomerase.

5. A mutant glucose isomerase enzyme according to Claim 1, characterized in that it distinguishes over a naturally occurring glucose isomerase having the same essential biological activity in that it has a K_m for xylose, at temperatures between 20 °C and 85 °C, lower than that of the naturally occurring glucose isomerase.

6. A mutant glucose isomerase enzyme according to Claim 1, characterized in that it distinguishes over a naturally occurring glucose isomerase having the same essential biological activity in that it has a V_{max}glucose/V_{max}xylose ratio better than that of the naturally occurring glucose isomerase.

7. A mutant glucose isomerase enzyme according to Claim 1, characterized in that it distinguishes over a naturally occurring glucose isomerase having the same essential biological activity in that it has a $K_m$xylose/$K_m$glucose ratio higher than that of the naturally occurring glucose isomerase.

8. A mutant glucose isomerase enzyme according to Claim 1 to 7, which is obtainable from a microorganism of the order of Actinomycetales.

9. A mutant glucose isomerase enzyme according to any one of Claims 1 to 8, which is derived from Actinoplanes missouriensis.

10. A mutant glucose isomerase enzyme according to any one Claims 1 to 7, in which the amino acid sequence of said glucose isomerase shows at least 65% homology with the amino acid sequence of the glucose isomerase derived from the wildtype Actinoplanes missouriensis strain.

11. A mutant glucose isomerase according to claims 1-7, which has a substitution of residues within a sphere of 4 angstroms around the oxygen atoms of a sugar substrate.

12. A mutant glucose isomerase according to claim 1-7 in which at least one of the following amino acids has been substituted :
15Leu, 17Thr, 20Trp, 25Ala, 27Gly, 52Thr, 53Phe, 55Asp, 57Asp, 87Pro, 88Met, 89Val, 90Thr, 91Thr, 92Asn, 93Leu, 95Thr, 133Thr, 134Leu, 135Val, 136Leu, 138Gly, 140Arg, 179Ala, 180Ile, 182Pro, 186Glu, 215Asn, 216Pro, 218Thr, 219Gly, 243His, 244Ile, 246Leu, 254Phe, 256Gl, 257Asp, 258Leu, 290His, 291Phe, 293Tyr, 294Lys, 295Pro and which has an altered substrate specificity.

13. A mutant according to claim 12, characterized in that it contains at least one of the following amino acid substitutions : A25K, M88S, T90S, V135Q, V135T, E186D, E186Q, N215S, H243N, K253R, L258K, H290N, K294Q, K294R.

14. A mutant according to claim 1-7 obtained by shifting water molecules in a sphere of 4 angstroms around

the oxygen atoms of a sugar substrate.

15. A method for obtaining an enzyme with an altered substrate specificity by changing an amino acid which is selected according to a method comprising the following criteria :
    a) select all residues and crystallographically assigned water molecules which have at least one atom within a sphere of 4Å surrounding the atoms of the substrate or of a substrate analogue/inhibitor bond in the active site ;
    b) select all the residues which are in Van der Waals contact with the residues and water molecules obtained by the selection according to criterion a) ;
    c) discard from the recorded list of residues and water molecules those that are implied in catalysis, cofactor binding (such as metal ions and nucleotides) and essential intersubunit interactions in the case of oligomeric enzymes ;
    d) discard those residues and water molecules that interfere with the structural role of these residues. Model building and analysis of the conserved nature of the target residues can be used to identify an essential structural role.

16. A method for obtaining an enzyme with an altered substrate specificity according to Claim 15, further characterized in that one or more of the following interactions and properties are changed :
    – e1) steric hindrance through altering the residue size ;
    – e2) hydrophobicity/polarity of the substrate surroundings ;
    – e3) solvation of the substrate surroundings either by providing side chains that solvate via hydrogen bonding to groups within the substrate or through substitutions that alter the water distribution within the surroundings of the substrate ;
    – e4) flexibility of individual residues, segments or the overall protein structure by substitutions that disrupt hydrogen bonding networks, decrease the local packing density in the surroundings of the substrate or introduce cavities.

17. A process for obtaining a mutant glucose isomerase molecule according to any one of claims 1-13 comprising :
    a) obtaining a DNA sequence encoding a glucose isomerase,
    b) mutating this sequence at selected nucleotide positions,
    c) cloning the mutated sequence into an expression vector in such a way that the DNA sequence can be expressed,
    d) transforming a host organism or cell with said vector,
    e) culturing said host organism,
    f) isolation the glucose isomerase.

18. Use of a mutant glucose isomerase according to any one of the preceding claims in the conversion of sugar molecules.

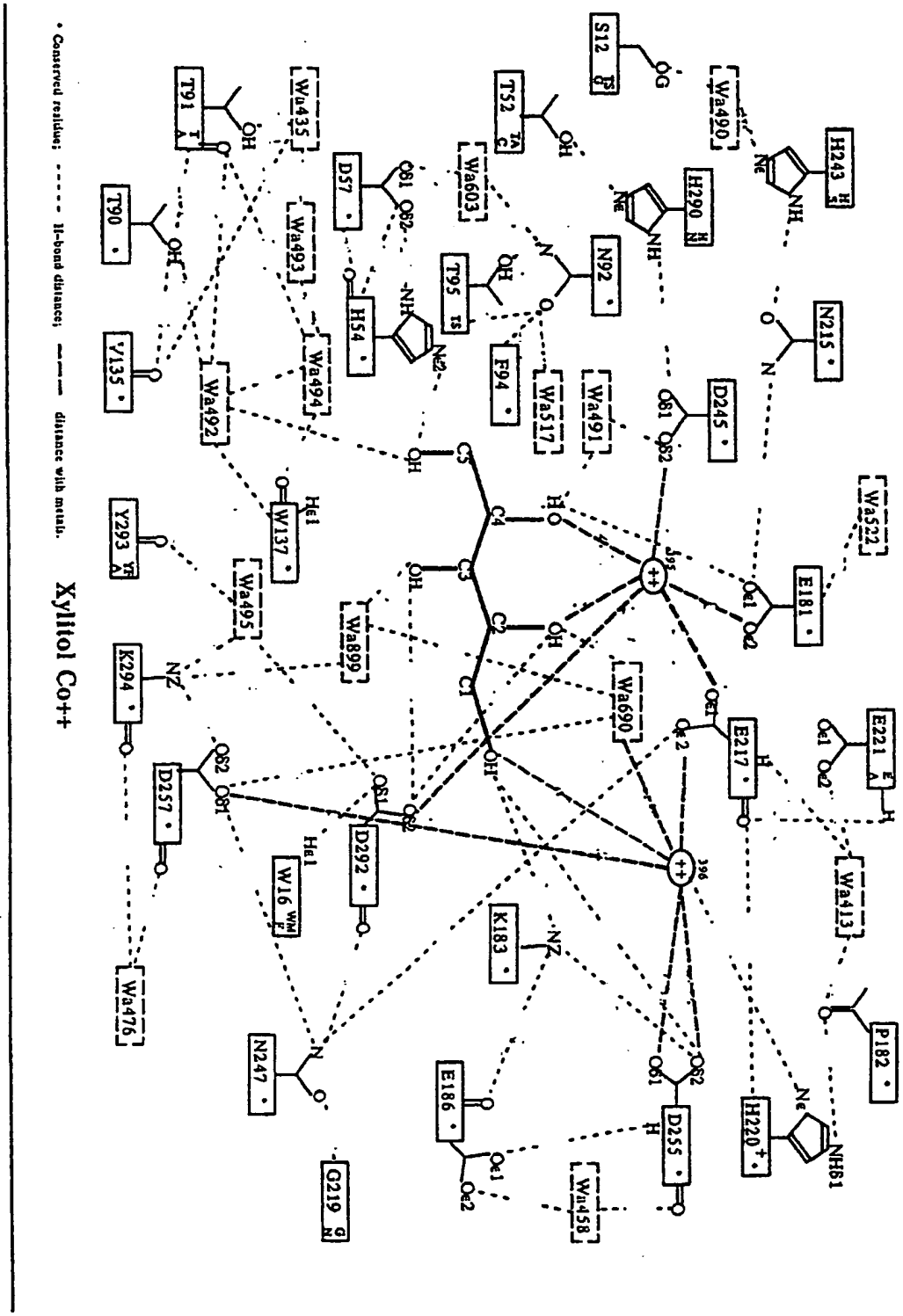
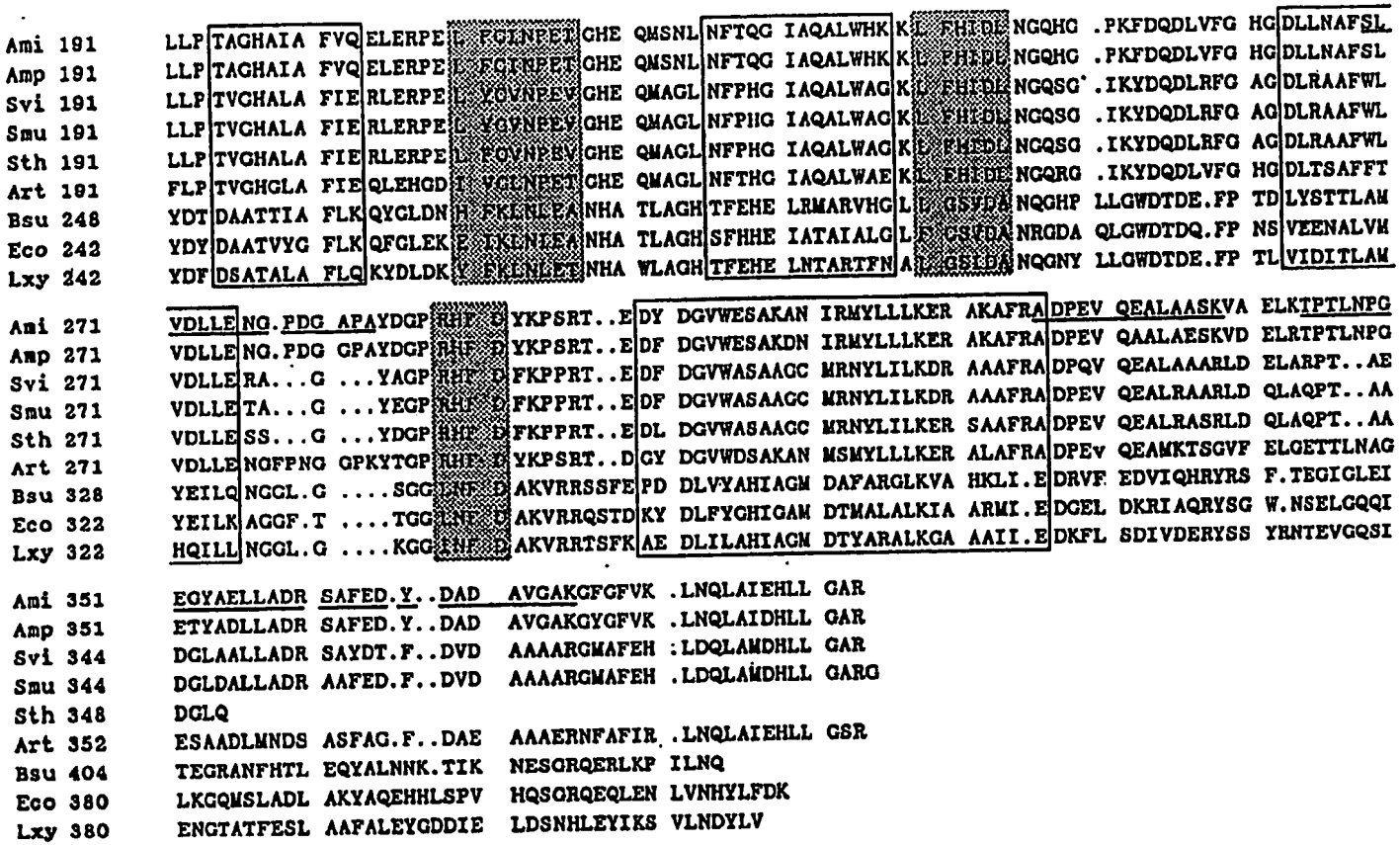

Xylitol Co++
* Conserved residues; ------ H-bond distance; ——— distance with metals.

FIGURE 1

FIGURE 2A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Sth 191 LLP TVGHALA FIE RLERPE L FGVNPEV GHE QMAGL NFPHG IAQALWAG KL FHIDL NGQSG .IKYDQDLRFG AG DLRAAFWL
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Bsu 248 YDT DAATTIA FLK QYGLDN H FKLNLEA NHA TLAGH TFEHE LRMARVHG LL GSVDA NQGHP LLGWDTDE.FP TD LYSTTLAM
Eco 242 YDY DAATVYG FLK QFGLEK F IKLNLEA NHA TLAGH SFHHE IATAIALG LF GSVDA NRGDA QLGWDTDQ.FP NS VEENALVM
Lxy 242 YDF DSATALA FLQ KYDLDK Y FKLNLET NHA WLAGH TFEHE LNTARTFN AL GSLDA NQGNY LLGWDTDE.FP TL VIDITLAM

Ami 271 VDLLE NG.PDG APAYDGP RHF G YKPSRT..E DY DGVWESAKAN IRMYLLLKER AKAFRA DPEV QEALAASKVA ELKIPTLNPG
Amp 271 VDLLE NG.PDG GPAYDGP RHF D YKPSRT..E DF DGVWESAKDN IRMYLLLKER AKAFRA DPEV QAALAESKVD ELRTPTLNPG
Svi 271 VDLLE RA...G ...YAGP RHF D FKPPRT..E DF DGVWASAAGC MRNYLILKDR AAAFRA DPQV QEALAAARLD ELARPT..AE
Smu 271 VDLLE TA...G ...YEGP RHF D FKPPRT..E DF DGVWASAAGC MRNYLILKDR AAAFRA DPEV QEALRAARLD QLAQPT..AA
Sth 271 VDLLE SS...G ...YDGP RHF D FKFPRT..E DL DGVWASAAGC MRNYLILKER SAAFRA DPEV QEALRASRLD QLAQPT..AA
Art 271 VDLLE NGFPNG GPKYTGP RHF D YKPSRT..D GY DGVWDSAKAN MSMYLLLKER ALAFRA DPEV QEAMKTSGVF ELGETTLNAG
Bsu 328 YEILQ NGGL.G ....SGG LNF D AKVRRSSFE PD DLVYAHIAGM DAFARGLKVA HKLI.E DRVF EDVIQHRYRS F.TEGIGLEI
Eco 322 YEILK AGGF.T ....TGG LNF D AKVRRQSTD KY DLFYGHIGAM DTMALALKIA ARMI.E DGEL DKRIAQRYSG W.NSELGQQI
Lxy 322 HQILL NGGL.G ....KGG LNF D AKVRRTSFK AE DLILAHIAGM DTYARALKGA AAII.E DKFL SDIVDERYSS YRNTEVGQSI

Ami 351 EQYAELLADR SAFED.Y..DAD AVGAKGFGFVK .LNQLAIEHLL GAR
Amp 351 ETYADLLADR SAFED.Y..DAD AVGAKGYGFVK .LNQLAIDHLL GAR
Svi 344 DGLAALLADR SAYDT.F..DVD AAAARGMAFEH :LDQLAMDHLL GAR
Smu 344 DGLDALLADR AAFED.F..DVD AAAARGMAFEH .LDQLAMDHLL GARG
Sth 348 DGLQ
Art 352 ESAADLMNDS ASFAG.F..DAE AAAERNFAFIR .LNQLAIEHLL GSR
Bsu 404 TEGRANFHTL EQYALNNK.TIK NESGRQERLKP ILNQ
Eco 380 LKGQMSLADL AKYAQEHHLSPV HQSGRQEQLEN LVNHYLFDK
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FIGURE 2B

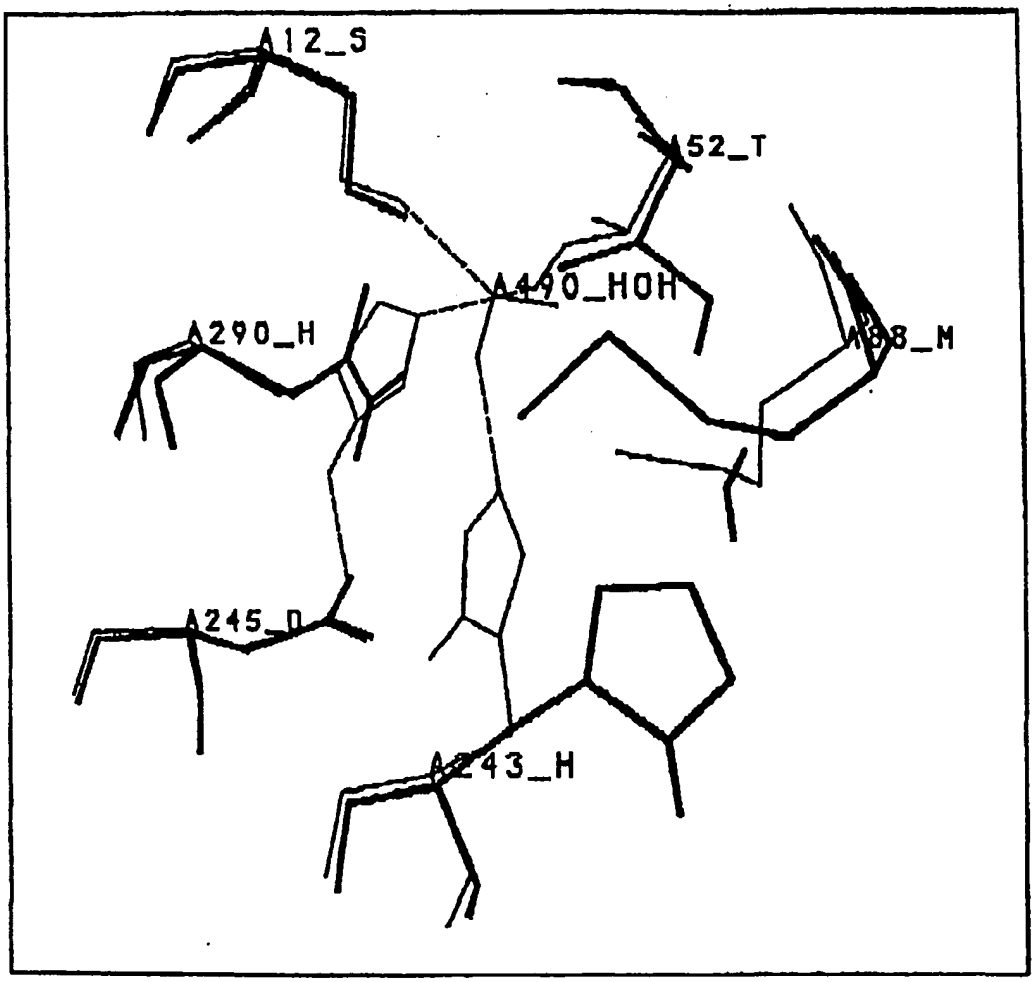

FIGURE 3